ESTROGENS IN MARINE INVERTEBRATES

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Material with estrogenic activity demonstrable by bioassay in rodents has been found in marine invertebrate tissues. Steidle (1930), using a mouse bioassay, found traces of estrogens in a sea urchin, *Echinus miliaris,* three molluscs, *Aplysia, Octopus* and *Eledone,* as well as in certain worms and arthropods. Similarly Schwerdtfeger (1932) found estrogens in a sea anemone, *Actinia aquina,* but none in the mollusc *Chiton marginatus.* Donahue (1940) reported small amounts of estrogenically active material to be present in extracts of the Bermuda urchin, *Lytechinus variegatus,* the reef urchin, *Echinometria,* a holothurian, *Stichopus mobii,* and a lobster, *Palinurus argus.* More recently, this same author (Donahue, 1948) made extracts of the shed eggs of another lobster, *Homarus americanus,* purified them by solvent partition, and made estrogen analyses by a fluorometric method. In this way he found 100 international units of estrogen per 100 gm. eggs.

Gordon and Villee (1956) have described an enzymatic assay for estrogens, which is as sensitive as the fluorometric methods now available. Their assay depends upon the fact that human placenta contains a DPN(diphosphopyridine nucleotide)-linked isocitric dehydrogenase which catalyzes the reaction.

$$\text{isocitrate} + \text{DPN} \rightleftharpoons \alpha\text{-ketoglutarate} + \text{DPNH} + CO_2$$

and which is specifically activated by certain natural estrogens. In a limited range the degree of enzyme activation is a function of the amount of hormone present. Since they measured the appearance of reduced DPN spectrophotometrically, it was essential that the material being assayed give a clear solution, and for that reason they found it impossible to analyze blood and tissue extracts. The progress of the reaction can also be measured chemically, by analyzing the reaction mixture after incubation for total keto-acids produced, and in this way relatively impure tissue extracts can be assayed. Such assays done on the ovaries of a number of marine invertebrates are reported here.

Methods

Invertebrate tissues were extracted for assay by the procedure used by Folch *et al.* (1954) for preparing total lipide extracts. The tissue was removed from the animal, blotted gently with filter paper, weighed accurately and homogenized in a Waring blendor containing 20 ml. 2:1 chloroform:methanol per gram of tissue. The homogenate was filtered and the residue discarded. The filtrate was washed

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once with 0.2 volume of distilled water and once with 0.2 volume of 0.01% aqueous calcium chloride which had previously been equilibrated with 2:1 chloroform:methanol. The extract was evaporated to dryness at room temperature under a gentle stream of clean air. The residue was taken up in ethanol for analysis and if, after thorough mixing, the solids did not go completely into solution, they were allowed to settle and the clear alcoholic extract was used for assay.

A 20% w/v homogenate of term human placenta was made in ice-cold 0.25 *M* sucrose, and centrifuged for 10 minutes at 600 G to remove cell nuclei and debris. The supernatant was then centrifuged at 57,000 G for 60 minutes to remove cellular particulate matter (Villee, 1955). The supernatant from the latter centrifugation contains the soluble matter of the cell, including the DPN-linked isocitric dehydrogenase. The enzyme-catalyzed reaction was carried out in 20-ml. beakers in a Dubnoff incubator at 37°, and was allowed to proceed for one hour. Each reaction vessel contained one ml. of the placental enzyme preparation; one ml. buffer containing 30 micromoles K^+, 10 micromoles Mg^{++}, 20 micromoles phosphate, and 20 micromoles Cl^-, adjusted to pH 7.3; 0.9 ml. water containing 0.75 micromole DPN and 3 micromoles cis-aconitate; and 0.1 ml. ethanol in which the standard or unknown solution was dissolved. The reaction was started by adding the DPN. Crystalline estradiol-17β was used as a standard. Analyses of the reaction mixture for total keto-acid production were made by a modification of the method of Friedemann and Haugen (1943). The amount of keto-acid produced in vessels containing all components except estradiol was used as a blank correction for the standard, and that produced in vessels containing all components except DPN was used as a blank correction for the unknowns. Total nitrogen analyses of the enzyme preparation were made by a micro-Kjeldahl procedure. The keto-acid analysis results were calculated in micromoles keto-acid produced per milligram nitrogen per hour. Duplicate reaction vessels were incubated and analyzed for each of the standards and unknowns in each assay.

Results

Separate standards were assayed with each set of unknowns, and the average results from 12 sets of standards are plotted in Figure 1. The least squares curve for these points is also shown. There is clearly a linear relationship between the logarithm of the amount of estradiol added and the amount of keto-acid produced over the range from 0.05 to 0.25 microgram estradiol per vessel. The index of precision, (λ), for this curve is 0.3 (Bliss, 1944). Recoveries of estradiol added to tissue before extraction averaged 99%.

The amount of estrogen in each unknown was calculated from a simultaneously determined standard curve for the same placental enzyme preparation. Each tissue extract was assayed with at least three different enzyme preparations from three different placentas. Of the tissues extracted, only the ovaries of *Mactra* (*Spisula*) *solidissima* contained significant amounts of estrogenic material, 1.1 $\pm$ 0.4 (mean $\pm$ standard error) milligrams estradiol equivalents per kilogram of fresh tissue. The other tissues, including the ovaries of *Asterias forbesi*, *Arbacia punctulata*, *Strongylocentrotus droebachiensis*, *Loligo pealei*, *Busycon canaliculatum*, *Carcinides maenas*, *Homarus americanus*, and *Limulus polyphemus*, the whole tissue of *Microciona prolifera* and the liver of *Homarus americanus* all contained less than 50 micrograms estradiol equivalents per kilogram of fresh tissue.

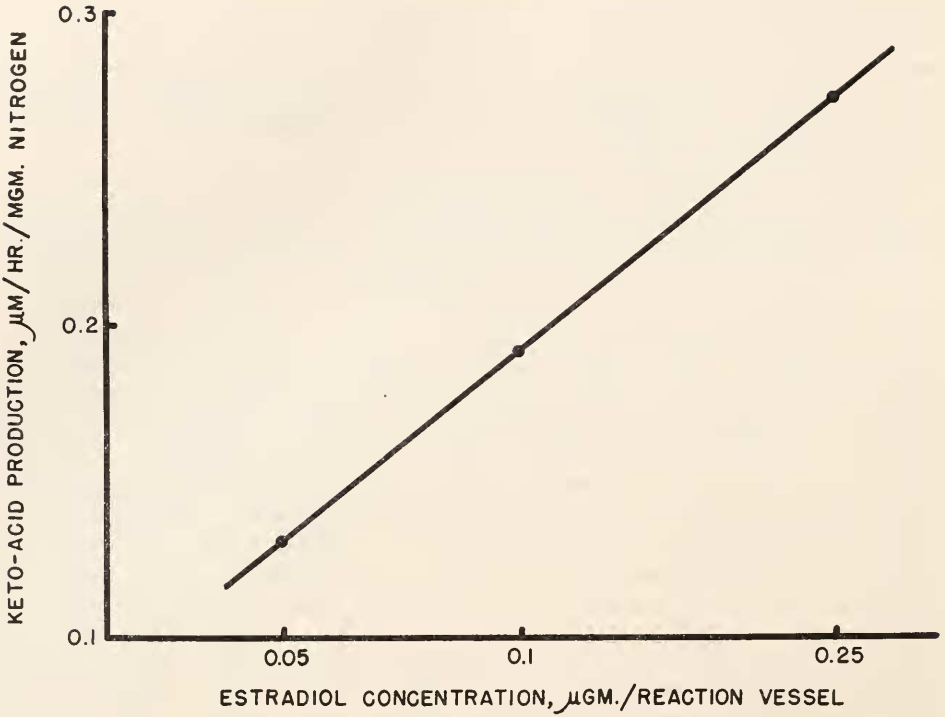

FIGURE 1. Keto-acid production by placental isocitric dehydrogenase as a function of estradiol-17β concentration.

DISCUSSION

The results were calculated in terms of estradiol equivalents, since that compound was used as a standard. It has been shown (Villee and Gordon, 1956) that estrone, equilenin and equilin are as effective as estradiol in stimulating the enzyme, while a variety of other steroids and synthetic compounds do not affect the enzyme unless present in very large quantities. It is thus likely that the material present in *Mactra* ovary is one of these four compounds.

The minimum amount of estrogen that can be detected in this assay is about 0.01 microgram of estradiol, and the tissue concentration that can be accurately determined depends, of course, on the amount of tissue extracted. With samples of tissue of about 10 grams, the analytical blanks are not unduly large and this magnitude of sample was used in these experiments. In all the tissues examined except *Mactra* ovary, the results of the assays indicated the presence of small amounts of active material, confirming the experience of earlier workers. The amounts are probably much smaller than the upper limit of 50 micrograms per kilogram which the present results make certain, but it would be necessary to extract larger quantities of tissue to make accurate estimates of the true amount present.

The precision of which this assay method is capable depends on the same factors as any other bioassay, the index of precision of the standard curve and the number

of replicates which are made. The former is satisfactory and the effort expended in making many replicates by this method is less than with many other forms of bioassay. Even with the small number of assays that were done in the present work on the *Mactra* ovary extracts, one can be quite confident that this tissue contains a very large amount of estrogenic material, presumably estradiol or a closely related compound. One milligram per kilogram is ten times as much as the maximum previously reported (lobster eggs) for any species. Estradiol added *in vitro* has no effect on the metabolism of *Mactra* ovary (Hagerman, 1956) and apparently no physiologic effects of estrogens on molluscs have been described. The material may be present as an evolutionary freak, similar to the occurrence of uric acid as an excretory product in the Dalmatian coach hound, or may have some important physiologic function in this mollusc. Further speculation should await the isolation and complete chemical characterization of the material present in *Mactra*.

Summary

1. An enzymatic method of assay for estrogens suitable for use with crude tissue extracts is described.

2. Of a variety of marine invertebrates examined, only the ovaries of the mollusc, *Mactra* (*Spisula*) *solidissima*, contained appreciable amounts of estrogenic material.

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